

HIV Infection Rapidly Induces and Maintains a Substantial Suppression of Thymocyte Proliferation

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Summary

The supply of naive T cells by the thymus normally requires precursor T cell proliferation within the thymus and would be particularly important in the setting of HIV infection when both naive and memory T cells are progressively depleted. As a robust, quantitative index of intrathymic proliferation, the ratio of different

T cell receptor excision circles (TRECs), molecular markers of distinct T cell receptor rearrangements occurring at different stages of thymocyte development, was measured in peripheral blood-mononuclear cells (PBMCs). This ratio has the virtue that it is a “signature” of thymic emigrants throughout their entire life and, thus, can be measured in peripheral cell populations that are easy to obtain. Using the new assay, we evaluated the effect of HIV infection on intrathymic precursor T cell proliferation by longitudinal analysis of PBMCs from recently infected individuals. Our findings reveal a substantial reduction in intrathymic proliferation. The analysis also indicates the existence of a compensatory mechanism acting to sustain the numbers of recent thymic emigrants (RTEs) in the periphery.

Introduction

HIV infection leads to sustained immune activation and to major alterations in T cell homeostasis (Douek, 2003; Roederer et al., 1997; Silvestri and Feinberg, 2003). In particular, naive cells, CD4⁺ and CD8⁺ alike, are progressively depleted (Roederer et al., 1997), possibly as a consequence of their frequent activation and differentiation into memory cells (Hazenberg et al., 2000a). Because the division of naive T cells is normally minimal and remains quite low in HIV-infected persons (Hazenberg et al., 2000c; Sachsenberg et al., 1998), their maintenance may critically depend on input from the thymus (Douek, 2003; Tanchot and Rocha, 1997). Thymic function may, however, be impaired in HIV infection (Douek et al., 2001; Hatzakis et al., 2000; Richardson et al., 2000). It has been difficult to assess the extent of such impairment (Aladdin et al., 2003; Douek et al., 2001; Hazenberg et al., 2000a, 2000b).

The difficulty is due to the lack of direct measures of thymopoiesis. In the thymus, both T cell receptor (TCR) chain loci, TCR α and TCR β , undergo rearrangement of their different gene segments, ultimately leading to the generation of highly diverse CDR3 regions. Byproducts of these processes, TCR excision circles (TRECs), persist in recent thymic emigrants (RTEs) as episomal DNA circles (Douek et al., 1998; Kong et al., 1999; Livak and Schatz, 1996; Poulin et al., 1999). The sjTRECs, generated through excision of the TCR δ locus, a prerequisite to TCR α chain rearrangement, have been extensively studied in blood T cells as a surrogate marker for thymic function (Aladdin et al., 2003; Douek et al., 2001; Geenen et al., 2003; Hazenberg et al., 2001; Ometto et al., 2002; Poulin et al., 2003; Steffens et al., 2001; Teixeira et al., 2001). However, because TRECs are not regenerated upon subsequent division of TREC-containing cells, their frequency within the T cell population reflects, in part, past proliferation experience of the population in the periphery and therefore is hard to interpret (Hazenberg et al., 2000b).

Because it has been demonstrated (Almeida et al., 2001) that the level of thymic output is primarily deter-

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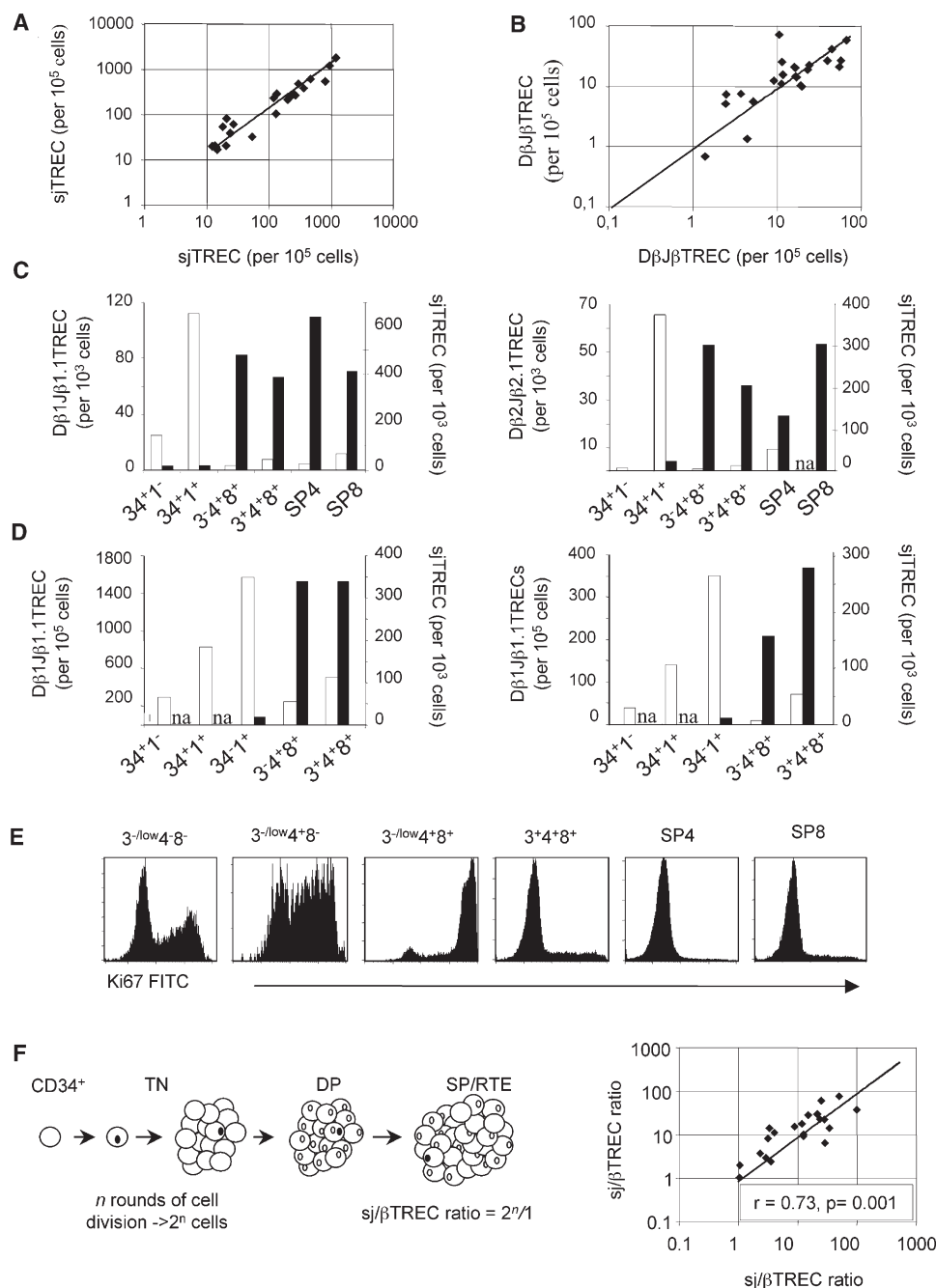


Figure 1. Quantification of sjTREC and DβJβ TREC Frequencies

(A–B) The sjTREC and DβJβ TREC frequencies were measured in duplicate in a group of 20 samples (seven from healthy controls and 13 from HIV-infected patients). The correlation between the two quantifications (Spearman's, $r = 0.959$ and $p < 0.0001$ for the sjTREC and $r = 0.754$ and $p = 0.001$ for the DβJβ TRECs) and the fact that the regression curves are close to the diagonal demonstrate the reproducibility of these assays.

(C and D) Evolution of DβJβ TREC and sjTREC frequencies during thymopoiesis. sjTREC frequencies (closed bars) and DβJβ TREC frequencies (open bars) were quantified by real-time quantitative PCR on FACS-purified thymocyte subpopulations as defined by CD34, CD1, CD3, CD4, and CD8 surface expression. Representative examples of two out of eight pediatric thymii (C) and two out of three adult thymii (D) are presented. The DβJβ TRECs and sjTREC were quantified in duplicates in each thymocyte subpopulation for each individual.

(E) Intrathymic proliferation was assessed by intracellular Ki67 expression on thymocyte subpopulations as defined by surface CD3, CD4, and CD8 expression by flow cytometry. A minimum of 3×10^5 events in the live cell gate as defined by forward and side scatter was accumulated for each sample.

(F) Schematic model of the generation of the sj/βTREC ratio as a measure of intrathymic proliferation. DβJβ TRECs (closed circles) are generated in TN cells ($CD34^{+}CD1^{+}CD3^{-}CD4^{-}CD8^{-}$), whereas the δ Rec- β J α rearrangement, leading to the generation of sjTRECs (open circles), occurs in DP ($CD3^{+}CD4^{+}CD8^{+}$). In between, thymocytes extensively proliferate, increasing the sj/βTREC ratio.

(G) The sj/βTREC ratio was measured in duplicate in a group of 20 samples (seven from healthy controls and 13 from HIV-infected patients). The correlation between the two quantifications (Spearman's, $r = 0.733$ and $p = 0.001$) and the fact that the regression curve is close to the diagonal demonstrate the reproducibility of the assay.

mined by the intrathymic proliferation of precursor T cells, we have developed an assay from which the relative changes in intrathymic proliferation can be readily and reliably estimated. This assay extends the use of TRECs to those associated with TCR β as well as TCR α gene rearrangements, circumventing some of the inherent interpretational difficulties encountered previously in attempting to assess thymic function by enumerating only sjTRECs. In particular, our estimates are not significantly affected by variable proliferation and death rates of T cells in the periphery, allowing quantitative evaluation of intrathymic proliferation even in HIV-infected patients demonstrating heightened T cell turnover.

Results

The sj/ β TREC Ratio Mirrors Intrathymic Proliferation
During thymopoiesis, rearrangements at the TCR α , β , and δ loci lead to the generation of various types of TRECs. These molecules can be quantified in peripheral blood by nested quantitative real time PCR technology. This methodology allows accurate quantification of both sjTRECs, the major byproducts of excision of the TCR δ locus, and D β J β TRECs, created during the first step of TCR β locus reorganization, namely, the D β -J β rearrangement (Figures 1A and 1B).

Quantification of D β J β TRECs in sorted subsets of pediatric ($n = 8$) and adult ($n = 3$) thymocytes confirmed that TCR β chain rearrangement occurs within the CD34⁺CD1a⁺ compartment (Figures 1C and 1D). Later, δ Rec- ψ J α rearrangement, deleting the TCR δ locus, leads to the generation of the sjTREC molecule that appears at the CD3^{low}CD4⁺CD8⁺ stage of thymocyte development (Okamoto et al., 2002; Takeshita et al., 1989). In the process of T cell development, extensive proliferation of TCR β ⁺ α ⁺ cells precedes TCR α chain rearrangement that, in turn, leads to positive and negative selection (Huang et al., 2003; von Boehmer et al., 1999). This proliferation is a key determinant of thymic output (Almeida et al., 2001). Ex vivo staining thymocyte subsets with antibodies to Ki67, a nuclear antigen expressed in dividing cells and in cells that have recently divided, is consistent with other observations showing that thymocytes extensively proliferate between these two rearrangement events (Huang et al., 2003). Indeed, the bulk of thymocyte proliferation occurs between the CD3^{low}4⁺8⁺ and CD3^{low}4⁺8⁺ stages (Figure 1E). This proliferation leads to the dilution of D β J β TRECs, decreasing their proportion relative to that of sjTRECs in mature thymocytes undergoing positive and negative selection and, equivalently, in RTEs. Therefore, intrathymic precursor T cell proliferation, occurring between the two rearrangements, can be assessed in peripheral blood mononuclear cells (PBMCs) by measuring the sj/ β TREC ratio (sjTRECs divided by the sum of D β J β TRECs) (Figure 1F). In other words, the sj/ β TREC ratio is a direct measure of the intrathymic proliferation of TCR β ⁺ α ⁺ thymocytes. Duplicate estimation of the sj/ β TREC ratio in a panel of 20 samples demonstrates the reproducibility of such intrathymic proliferation assess-

ment (Spearman's rank, $r = 0.733$ and $p = 0.001$) (Figure 1G) based particularly on our ability to accurately measure the less frequent D β J β TRECs.

Peripheral T Cell Proliferation Does Not Influence the sj/ β TREC Ratio

SjTREC- and D β J β TREC-containing T cells in the periphery are functionally indistinguishable from each other and from those that do not contain TRECs. Therefore, the sj/ β TREC ratio in PBMCs should be independent of T cell proliferation. PBMCs from healthy individuals were activated in vitro with anti-CD3 antibody to confirm this. The sjTREC and D β J β TREC frequencies were measured daily in the cultures for one week. As demonstrated in Figures 2A–2C, the sj/ β TREC ratio remained constant during this period, whereas the sjTREC and D β J β TREC frequencies both decreased as the population expanded.

We also found no evidence that the sj/ β TREC ratio is influenced in vivo by T cell proliferation. The frequency of Ki67-expressing cells was quantified in various T cell subsets in healthy controls and in HIV-1-infected patients sampled during the first 6 months after infection. These were correlated to the sjTREC and D β J β TREC frequencies as well as to the sj/ β TREC ratio in the same samples (Figures 2D–2I). Negative correlation was observed in the patients' group between the frequency of proliferating naive T cells and that of either sjTREC or D β J β TREC frequencies ($r = -0.643$, $p = 0.02$ and $r = -0.852$, $p = 0.003$, respectively) (Figures 2E and 2G), possibly indicating a cumulative dilution effect that is related to the level of Ki67 expression. Indeed, the correlation of Ki67 expression with sjTREC frequencies was not significantly different than its correlation with D β J β TREC frequencies (multiple regression analysis, $p = 0.23$). Such a correlation is not observed in the control group (Figures 2D and 2F). Notably, we found no significant correlation between the sj/ β TREC ratio and Ki67 expression in any peripheral T cell subpopulation defined by the expression or absence of CD45RA and CD27, including the CD45RA⁺CD27⁺ naive T cells (Figures 2H and 2I), supporting the notion that this parameter is not significantly influenced by extrathymic proliferation. Because it is independent of peripheral T cell proliferation, foreign antigen-induced or homeostatic (Figure 2), the sj/ β TREC ratio is a reliable surrogate marker of intrathymic proliferation of precursor T cells even in pathological conditions such as during the course of HIV infection.

Age Dependence

In healthy individuals ($n = 28$), the sj/ β TREC ratio is age dependent (Spearman's, $r = -0.476$ and $p = 0.007$) (Figure 3A) and proportional to the sjTREC frequency (Spearman's, $r = 0.73$ and $p = 0.0001$) (Figure 3B), suggesting that both these parameters are directly linked to the level of intrathymic proliferation and indeed with thymic output, which declines with age. Although the peripheral frequency of sjTRECs decreases in parallel to age-associated thymic involution (Spearman's, $r = -0.527$ and $p = 0.003$) (Figure 3C), the frequency of D β J β TRECs does not (Figure 3D). The respective fre-

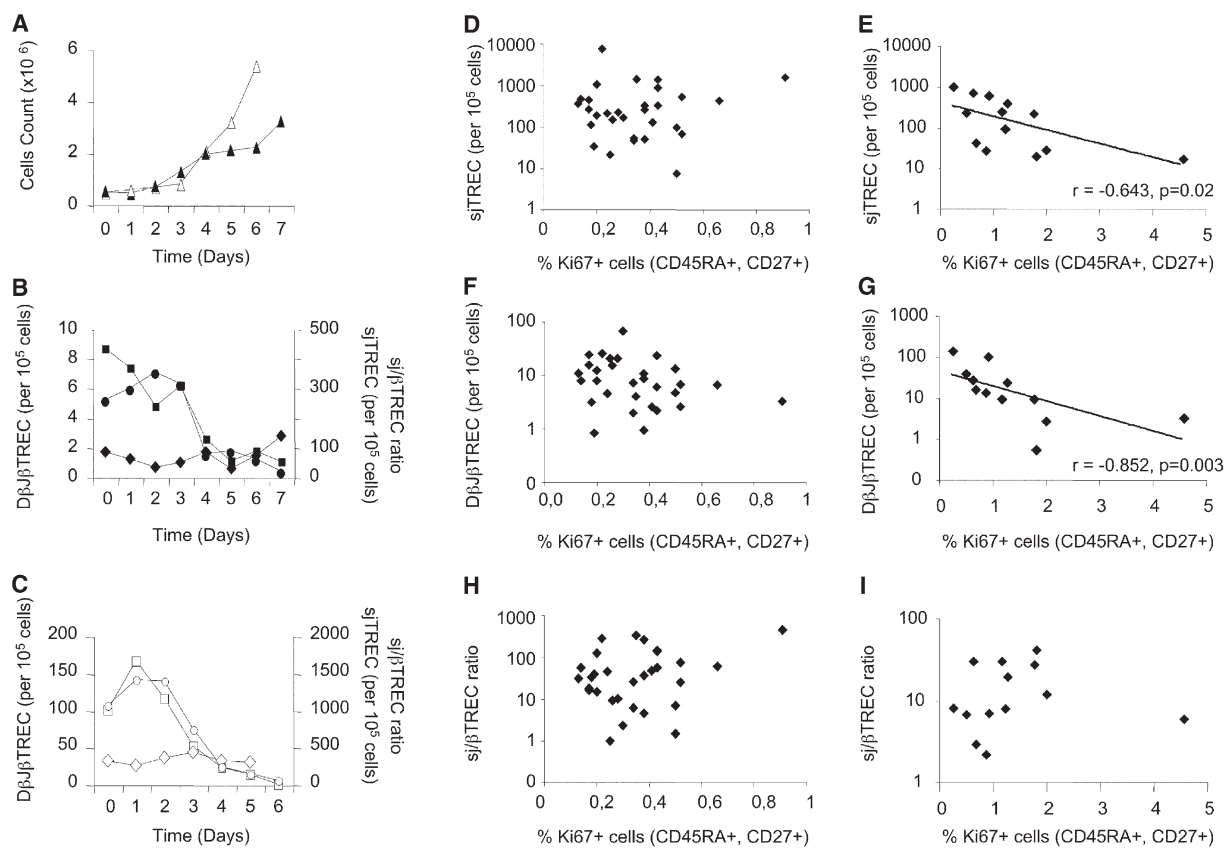


Figure 2. The sj/βTREC Ratio Is Not Dependent on Peripheral Proliferation

(A–C) Anti-CD3-stimulated PBMCs from two healthy individuals (open and closed symbols) were cultured for 1 week in the presence of IL-2. Every 24 hr, the total number of cells in the culture was measured (A). The sjTREC frequency ([B] and [C], squares), the DβJβ TREC frequencies ([B] and [C], circles), and sj/βTREC ratio ([B] and [C], diamonds) were quantified in duplicates for each individual time point.

(D–I) PBMCs from healthy individuals ([D], [F], and [H]; n = 28) or HIV-1-infected patients ([E], [G], and [I]; n = 12) were stained for surface markers CD3, CD45RA, and CD27 as well as for Ki67 nuclear antigen. The sjTREC (D and E) and DβJβ TREC (F and G) frequencies as well as the sj/βTREC ratio (H and I) are plotted as a function of Ki67 expression in naive T cells (CD3⁺CD45RA⁺CD27⁺). Negative correlation was observed in HIV-infected patients between the sjTREC frequency or the DβJβ TREC frequencies and naive T cell proliferation (Spearman's, $r = -0.643$, $p = 0.02$ and $r = -0.852$, $p = 0.003$ for the sjTREC and DβJβ TRECs, respectively).

quencies of these two TREC families within the thymus are, thus, differentially affected by aging.

Intrathymic Proliferation Is Rapidly Reduced after HIV Infection

The sj/βTREC ratio was measured longitudinally over 2 years in a group of 24 recently HIV-infected patients (1–6 months post infection) (see Supplemental Table S1 at <http://www.immunity.com/cgi/content/full/21/6/757/DC1>), before the initiation of antiretroviral therapy (ART), and in a group of 28 age-matched healthy individuals (Figure 4) to evaluate the impact of primary HIV infection (PHI) on intrathymic proliferation. Because of the limited availability of cells, total PBMCs were analyzed without further separating CD4 cells from CD8 cells or naive from memory cells. This does not affect our results and conclusions in any substantial way, because we trace intrathymic proliferation occurring before differentiation into the different subsets or later from the naive to the memory compartment. The sj/βTREC ratio is not affected by such differentiation events.

Although stable in healthy controls (Figure 4A), the sj/

βTREC ratio appears to have dropped within the first 6 months after HIV infection in the majority of untreated patients (17 of 24 studied). Fourteen patients had reduced ratios already at study entry (5- to 40-fold lower than the theoretical value calculated according to their age). In three patients, the sharp decline of the sj/βTREC ratio occurred after study entry (Figure 4B). In seven individuals, the sj/βTREC ratio did not appear to have changed. The postinfection sj/βTREC ratio, measured after the initial decline (median time postinfection 104 days, range 35–192), was significantly (Student's *t* test, $p = 0.0002$; Mann-Whitney, $p = 0.0007$) (Figure 3C) lower in patients (median 7.9, range 0.7–66, mean 14.9; $n = 24$) than in the age-matched controls (median 32.5, range 2–436, mean 78.2; $n = 28$). These results demonstrate that in most cases, intrathymic precursor T cell proliferation decreased rapidly and profoundly during the early phase of HIV infection.

Infection-Induced Decrease in TREC Ratio Is Age Dependent and Associated with DβJβTREC Increase Because thymic function is intimately linked to age, healthy controls and HIV-1-infected patients were sepa-

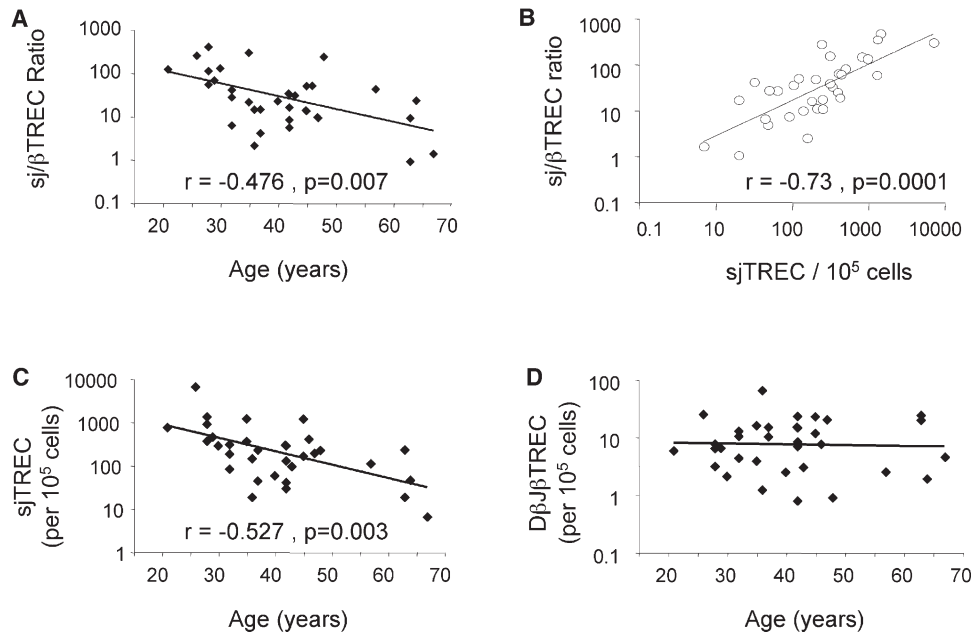


Figure 3. Characterization of the sj/βTREC Ratio as a Marker of Intrathymic Proliferation

(A) The sj/βTREC ratio (as defined by the sjTREC frequency divided by the sum of DβJβ TREC frequencies) was measured in the group of healthy individuals ($n = 28$). The sj/βTREC ratio was inversely proportional to age (Spearman's, $r = -0.476$ and $p = 0.007$).

(B) The sj/βTREC ratio and the sjTREC frequency were quantified in the group of healthy individuals ($n = 28$). The figure demonstrates a proportionality (Spearman's, $r = 0.73$ and $p = 0.0001$) between the two measurements.

(C and D) The sjTREC and DβJβ TREC frequencies were quantified in the group of healthy controls ($n = 28$). Although the sjTRECs inversely correlated with age (Spearman's, $r = -0.527$ and $p = 0.003$), there was no correlation between age and DβJβ TREC frequency.

rated into two age groups (≤ 40 and >40 years). As demonstrated in Figures 4d, 4E, and 4F, HIV-infected individuals under 40 had lower sjTREC levels than age-matched controls (median frequencies 220 and 449 per 10^5 cells in patients and controls, respectively; Student's t test, $p = 0.03$; Mann-Whitney, $p = 0.037$) (Figure 4D). By contrast, a significantly higher frequency of DβJβ TRECs characterized young HIV-infected patients as compared to any other group (median 24.6 and 6.8 per 10^5 cells in patients and controls, respectively; Student's t test, $p = 0.02$; Mann-Whitney, $p = 0.024$) (Figure 4E). These two opposite variations translate into a strong decrease in the sj/βTREC ratio in young patients as compared to age-matched controls (median ratios 7.9 and 96.8 per 10^5 cells in patients and controls, respectively; Student's t test, $p = 0.0001$; Mann-Whitney, $p = 0.0005$). Indeed, although sjTREC frequencies are significantly diminished, this reduction does not fully reflect the large change in the sj/βTREC ratio in the majority of patients. It is the combined effect of this decrease in sjTRECs and a concomitant increase in DβJβ TREC frequencies, at least in younger individuals (Figure 4E), that numerically accounts for the substantial decline in the ratio. Interestingly, the median and distribution of the sj/βTREC ratios in the group of younger patients are very similar to those measured in both infected and uninfected older subjects (Figure 4F). These data show that the impact of primary HIV infection on the dynamics of T cell precursors within the thymus is more significant in younger subjects, whose thymii are presumably more active before infection. It also shows that changes in individual TREC frequencies (and, presumably, in the

thymic output) are not simply predicted by the change in intrathymic proliferation (see Discussion).

Thymic Function Recovery after Antiretroviral Therapy

Having demonstrated that thymocyte proliferation is rapidly and profoundly impaired after HIV infection, especially in younger patients, we analyzed thymocyte proliferation in ten patients who initiated ART at an early stage (mean time from infection, 3 months [24–176 days]). All reached undetectable viral loads within the first 4 months of treatment along with stabilization of total CD4 counts and reduction of circulating CD8 T cells (mean CD4 increase 93 cells per microliter, mean CD8 decline 866 cells per microliter). One patient (patient T1 of Figure 5A) did not experience any significant fall in sj/βTREC ratio prior to treatment despite high viremia. In this patient, intrathymic proliferation remained constant over the follow-up period. The other nine patients showed very low intrathymic proliferation before treatment as inferred from their low sj/βTREC ratios compared to the normal values corresponding to their age (indicated in Figure 5A as horizontal gray lines). Although intrathymic proliferation under efficient ART remained stable in four subjects over the follow-up period (patients T2 to T5 of Figure 5A), the other five normalized their sj/βTREC ratio after treatment, reaching levels similar to those of age-matched controls (mean increase, 8.6-fold [1.87–33.06]). Despite these changes in the sj/βTREC ratio, sjTREC levels remained stable or only slightly increased during treatment. Thus, in arithmetic terms, a decrease in DβJβ TRECs accounts for most of the observed sj/βTREC ratio increase in patients T6 to

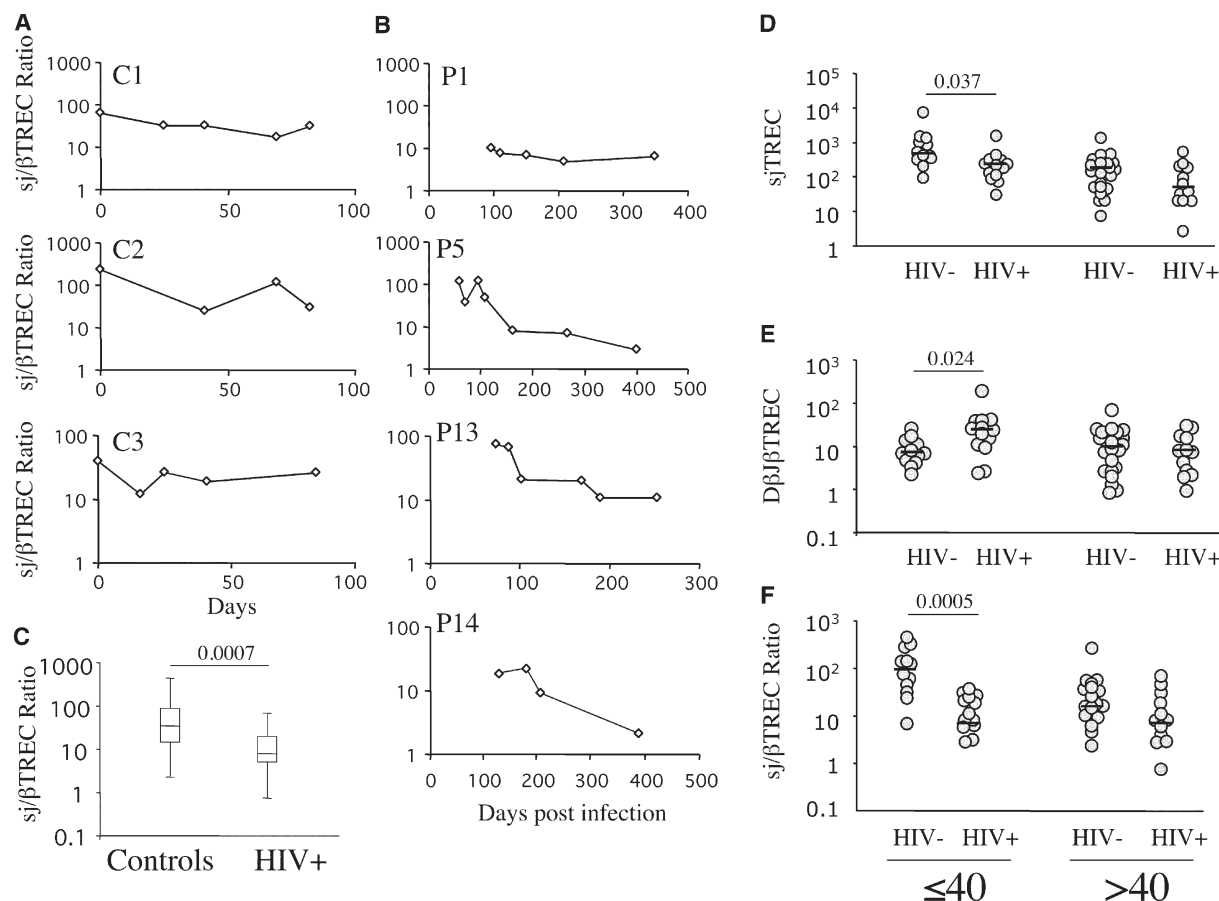


Figure 4. Age-Dependent Drop of the sj/βTREC Ratio during Primary HIV Infection

(A) The evolution of the sj/βTREC ratio was analyzed longitudinally in six healthy individuals sampled over a 3 month period. Representative examples are shown. The individuals were 24, 37, and 42 years old, from top to bottom.

(B) The evolution of the sj/βTREC ratio was analyzed in a group of 24 patients sampled during the first 2 years of infection. Examples are shown. The patients were 24, 30, 43, and 44 years old, from top to bottom.

(C) Box plot representation of the sj/βTREC ratio in untreated primary HIV-infected individuals. The sj/βTREC ratio was quantified by real-time quantitative PCR in a cohort of 24 patients, enrolled during early PHI and untreated at sampling time, and compared to healthy, age-matched controls (n = 28). The horizontal lines represent maximum, 75th percentile, median, 25th percentile, and overall minimum. Statistically significant difference between the two groups, calculated by Mann-Whitney test, is shown.

(D–F) HIV-infected patients sampled during primary infection and healthy controls were grouped by age (over and under 40 yrs old at sampling time). The sjTREC (D) and DβJβTREC (E) frequencies (per 10⁵ cells) as well as the sj/βTREC ratio (F) were measured for each sample. Statistically significant differences between groups, calculated by Mann-Whitney test, are shown above. 12, 12, 21, and 12 individuals were analyzed in HIV[−] ≤40, HIV⁺ ≤40, HIV[−] >40, and HIV⁺ >40 groups, respectively.

T10 (Figure 5B). Biologically, these data demonstrate that under ART, thymic function can be efficiently restored and other parameters consequently normalized.

Discussion

The ability to assess thymic function through quantification of sjTREC-containing cells among naive T cells in the periphery has been questioned given that the sjTREC frequency is directly influenced by the turnover of these cells, which is thought to be elevated in pathological conditions such as HIV infection (Hazenberget al., 2001). Indeed, we also observed such a correlation in the cohort of HIV-1-infected patients sampled during an early phase of chronic HIV infection (Figure 2).

This report describes a method of using TRECs to assess important aspects of thymic function that is not confounded by peripheral T cell division. We have added to the current available tools the measurement of a parameter, the sj/βTREC ratio that directly reflects precursor cell proliferation in the thymus, a key determinant of thymic output (Almeida et al., 2001). Furthermore, the sj/βTREC ratio has the virtue that it is a “signature” of thymic emigrants throughout their entire life and thus can be measured, unbiased, in total peripheral blood cells. We have evaluated the impact of HIV infection on this parameter and found a rapid sj/βTREC ratio drop (within 3 months postinfection), indicating a substantial decline in the proliferation of TCRβ⁺α⁺ precursor T cells. This reveals that HIV infection disrupts the development of T cells early in the course of disease progression.

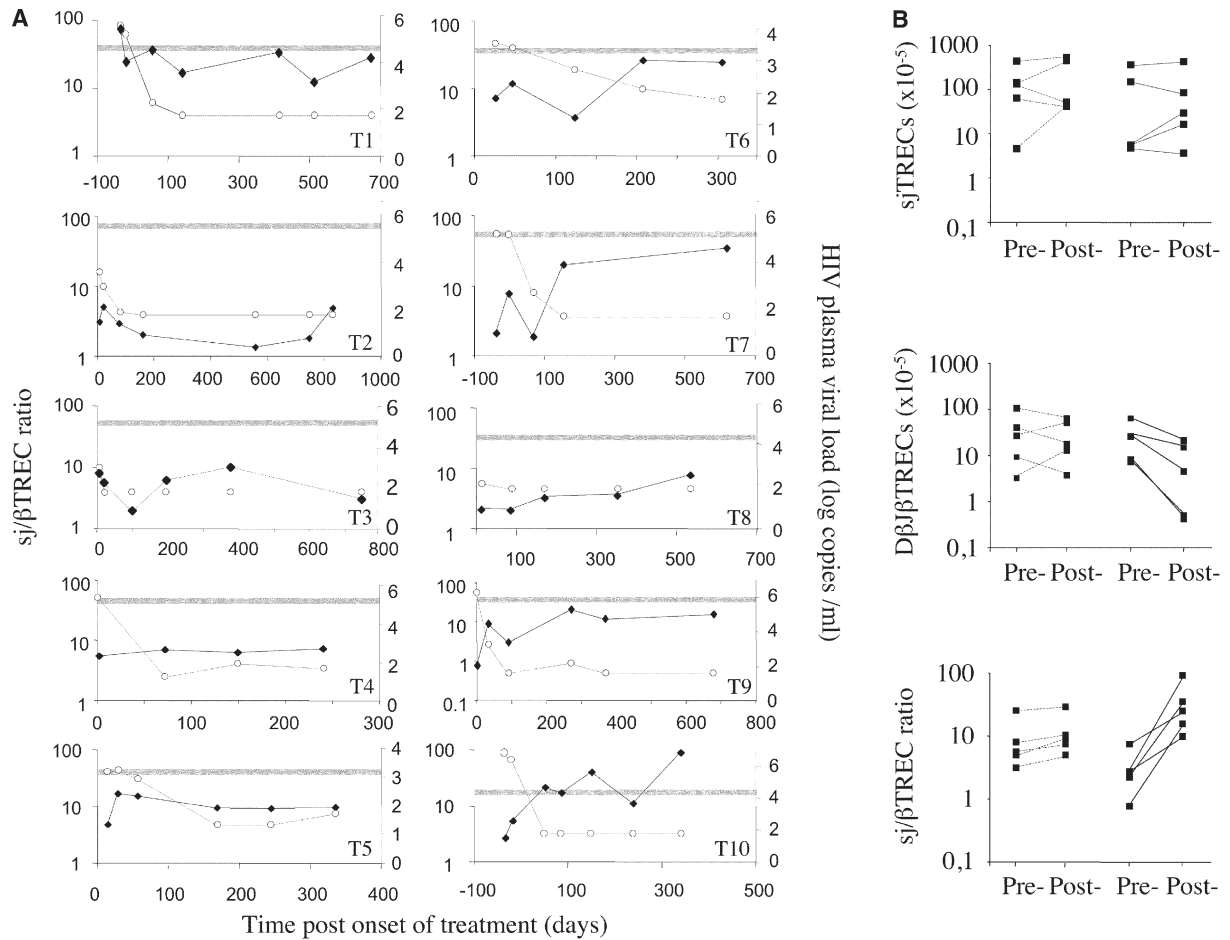


Figure 5. Effect of Early ART Administration on the sj/βTREC Ratio

(A) The sj/βTREC ratio was quantified in ten patients under ART. Plasma viral load (open circles) and sj/βTREC ratio (closed diamonds) are presented longitudinally over the follow-up period. Therapy was initiated 164 days or less postinfection. The horizontal line represents an estimated preinfection sj/βTREC ratio calculated according to the age of the patient with an empirical equation describing the age dependence of the sj/βTREC ratio in healthy individuals, derived from the equation of the correlation line defined in Figure 3A (theoretical sj/βTREC ratio = $613.46e^{-0.0681 \times \text{Age}}$).

(B) The sjTREC and DβJβTREC frequencies as well as the sj/βTREC ratio were measured prior to initiation of ART and after viral suppression. The post-ART values are given for the last analyzed time point. Patients T1 to T5 data are presented on the left, and T6 to T10 are presented on the right.

Mechanistically, the observed trend of increasing DβJβTREC frequencies in the studied HIV-infected patients suggests that direct killing of dividing thymocytes by the virus does not significantly contribute to the inability of these cells to proliferate normally. Killing would have resulted in a smaller output, not larger, of T cells containing DβJβTREC. Indeed, not only the frequency

but also the absolute number of these TRECs is larger than normal (see Table 1). Rather, the reduced intrathymic proliferation of T cell precursors might be a consequence of cytokine-mediated inhibition. For example, interferon α , produced as part of the innate immune response to infection, is known to inhibit the proliferation of peripheral T cells along the IL-7-p27 (Kip1) axis. These

Table 1. Evolution of Peripheral TREC Frequencies and Absolute Counts during Primary HIV Infection

	HIV ⁻ Controls	HIV ⁻ Controls ≤40	HIV ⁻ Controls >40	HIV ⁺ Patients	HIV ⁺ Patients ≤40	HIV ⁺ Patients >40
sjTREC/10e ⁵ cells	638 ± 1336	1161 ± 1933	247 ± 306	238 ± 347	360 ± 445	116 ± 147
DβJβTREC/10e ⁵ cells	12.0 ± 13.3	9.1 ± 6.9	14.1 ± 16.6	27.1 ± 44	43.8 ± 58	10.3 ± 10
sjTREC/ml	11,102 ± 26,737	23,211 ± 38,666	4182 ± 6130	4708 ± 7260	7243 ± 9489	2172 ± 2478
DβJβTREC/ml	237 ± 267	183 ± 137	268 ± 332	602 ± 941	890 ± 1232	314 ± 392
sj/βTREC ratio	80.2 ± 110.0	138.5 ± 135.4	31.9 ± 60.2	14.8 ± 16	7.91 ± 10.63	7.81 ± 20.35

The TREC frequencies and TREC numbers listed (TRECs/10e⁵ cells and TRECs/ml of blood) are the means ± standard deviations, measured in the different groups of subjects.

molecules are also involved in thymocyte proliferation (Kolluri et al., 1999).

It is intriguing that the input of RTEs after HIV infection, characterized by a different sj/ β TREC ratio, is sufficient to appreciably modify the overall peripheral sj/ β TREC ratio within a few weeks, considering that this input might be considerably smaller even compared to the normally small input. Indeed, it has been shown (Hazenberget al., 2000b) that changes in thymic output require a much longer time to substantially impact the size of the naive population. The relatively rapid kinetics of the TREC-containing T cells that we observed here suggests that in adult patients, RTEs are not only much richer in TRECs than any other T cells, including the bulk of the naive T cell population (Kimmig et al., 2002; McFarland et al., 2000), but also disappear much more rapidly; that is, most RTEs are short lived and are constantly replaced by new thymic emigrants. This too is consistent with earlier studies in mice (Berzins et al., 1999). Accordingly, the postinfection RTEs need only replace the majority of preexisting RTEs in the periphery, not the majority of all naive cells, to produce an overall change in the sj/ β TREC ratio.

These considerations shed new light on the age dependence of TREC frequencies that we observed in healthy adults (Figure 3). It had been previously argued that the age-related decline in sjTREC frequency is only an indirect measure of a declining thymic output, reflecting peripheral dilution because of a homeostatic increase in T cell proliferation (Hazenberget al., 2000b). The argument was based on a model assuming identical kinetics for RTEs and other naive cells (see also Grossman and Paul [2000]). Such dilution would have affected sjTRECs and D β J β TRECs in the same way. We, however, found that only the frequency of sjTRECs, but not that of D β J β TRECs, decreased with age along with the sj/ β TREC ratio. This finding leads us to two conclusions: first, that it is not the total throughput of thymocytes that is declining with age (as might be expected when a thymus becomes smaller) but, rather, the number of divisions per individual TCR β ⁺ α ⁺ thymocyte. The second conclusion is that the resultant decrease in the output of RTEs carrying sjTRECs is directly reflected in the peripheral frequency of these TRECs, consistent with the proposition that most TRECs are contained in a population of cells that have recently emerged from the thymus and that are being rapidly replaced.

Elevations in sjTREC and D β J β TREC frequencies that are not predictable by the reduction in the sj/ β TREC ratio reveal the existence of one or more compensatory mechanisms. Indeed, in younger HIV-infected patients, the sj/ β TREC ratio was 12-fold lower than in age-matched controls, and in these same individuals, the sjTREC frequency was reduced only 2-fold. Moreover, an opposite change in D β J β TREC frequencies was observed (3.5-fold increase). Thus, once intrathymic proliferation is impaired, as reflected by the strong reduction in TREC ratio, certain thymic and/or peripheral events subsequently occur that tend to prevent a proportional diminution in the number of the TREC-rich RTEs. In any event, it is clear that the sjTREC frequency or number is not primarily determined by a dilution effect of enhanced division rates of naive T cells, as was previously proposed. Such dilution would have resulted in a more

prominent, not less, sjTREC frequency decline relative to the decline observed in the sj/ β TREC ratio.

The sustained sjTREC and increased D β J β TREC frequencies in HIV-infected patients in the face of a reduced intrathymic proliferation, might reflect the operation of a feedback mechanism that controls thymic output (McCune et al., 1998) or the addition of RTEs to the general naive population. As a result, RTEs appear to be only moderately reduced in the early phase of HIV infection. The existence of a mechanism that enhances the production of T cells by the thymus in the younger age group of recently infected patients, possibly countering other effects that tend to diminish production, has been proposed by others for the CD4⁺ T cell subset only in a recent extensive study of conventional sjTRECs (Nobile et al., 2004). The direct relation between changes in sjTRECs and in RTEs implied by these authors can now be rationalized. Given the newly revealed kinetic dichotomy between RTEs and the established naive T cell population that we and others have discussed, factors that influence the production, survival, and/or dilution of RTEs can have a direct and relatively rapid impact on peripheral TREC frequencies. Possible factors are listed in Table 2 and their impact indicated. Such factors may include: (1) Thymus-filling effect. Once intrathymic proliferation is suppressed and the number of thymocytes declines, more bone marrow-derived cells may be allowed to enter the thymus as a result of a fall in "back pressure." This would act to increase the number of β -rearranged thymocytes, to partially normalize the thymic output and the peripheral frequency of sjTRECs, and to increase the peripheral D β J β TREC frequency (Table 2). (2) Enhanced survival of early thymocytes. The death rate of early thymocytes might depend on cell density. Enhanced survival in response to the suppression of intrathymic proliferation would have an effect similar to the thymus-filling effect. (3) Enhanced RTE survival. This would account for secondary increases in TREC frequencies in the periphery. Additionally it would partially compensate for a reduced thymic input owing to the intrathymic suppression of precursor cell division. (4) Enhanced turnover of naive T cells (Hazenberget al., 2000b). This can result only in a reduction of TREC frequencies and therefore, as has already been discussed, could not help in explaining the observed increases.

Unspecified changes in migration patterns of RTEs might also affect the observed frequencies (Chu et al., 2004). We note, on the other hand, that if reduced intrathymic proliferation extends to the last rounds of division at the late stage of intrathymic development, after TCR α rearrangement, this would further diminish the thymic output but would not affect the outflow of TREC-containing cells. Therefore, peripheral TREC frequencies should not be directly affected. Finally, in addition to the above-listed potential changes in the dynamics of TREC-carrying cells after HIV infection, apparent changes in TREC frequency (TREC number per a fixed number of T cells) would arise in HIV-infected individuals because of changes in the T cell count, even if the number of TREC-rich RTEs remained the same. Such an effect would be irrelevant to the issue of thymic function. Indeed, in the most extensive study to date on the impact of HIV infection on frequencies of sjTRECs in blood,

Table 2. Modifications of Thymocyte/RTE Dynamics after HIV Infection: Effect on Peripheral TREC Frequencies and Ratio

	Healthy Individuals	HIV-Infected Patients	(A) Increased Peripheral Proliferation	(B) Decreased Intrathymic Proliferation	(C) Increased Input into the Thymus	(D) Increased Thymocyte Survival	(E) Increased RTE Survival	Combined Effect ^a
sjTRECs	449	220 ^c	down ^b	down	up	up	up	moderately down
DβJβTRECs	6.8	24.6 ^c	down ^b	no effect	up	up	up	up
sj/βTREC ratio	96.8	7.9 ^d	no effect	down	no effect	no effect	no effect	down

The TREC frequencies listed (TRECs/10⁵ cells) are the medians over the <40-year-old patients studied. Note that the mean sj/βTREC ratio is not identical to the ratio of the mean TREC frequencies.

^a Combination of (B) with (C), (D), and/or (E).

^b Inconsistency with the data in the left columns.

^c Significant differences ($p < 0.03$).

^d Significant difference ($p = 0.0001$).

a large difference between the two subsets has been reported (Nobile et al., 2004) and most of this difference can be attributed to the relative (opposite) changes in CD4 and CD8 T cell counts that rapidly occurred after infection with HIV. In the present study, the “arithmetic” effect is nonsubstantial, because total T cell counts do not change much after HIV infection though CD4 and CD8 counts individually do. Nevertheless, to avoid the artifact of changes resulting from modified (total) T cell counts and also because TREC numbers are not directly affected by peripheral division, we converted all our observed TREC frequencies into TRECs/ml, and our conclusions remain the same (Table 1). As for the sj/βTREC ratio, we note that it is not only the ratio of the frequencies but also the ratio of total TRECs.

The question of how total numbers of TRECs change in specific cell populations is certainly of interest, though beyond the scope of the present study. In particular, it has been reported that in younger HIV-infected patients, not only the frequency but also the number of sjTRECs found in CD4⁺ T cells tended to be higher than normal, not lower (Nobile et al., 2004). Because cell counts in the HIV⁻ controls were not available, it was difficult to assess the statistical significance of the difference (J. Borghans, personal communication). Moreover, the authors have cautioned that the apparent increase in CD4 TREC numbers in young HIV patients with relatively high CD4 cell counts might have resulted from high presero-conversion CD4 TREC contents in this group of patients (Nobile et al., 2004); a longitudinal study is required to overcome this caveat. Differences in TREC numbers between the subsets can be expected, because the TREC-rich RTEs may become represented in the total population in different proportions depending on whether they are CD4⁺ or CD8⁺ cells because of differential division and/or survival rates after or just before leaving the thymus, as has been reported in mice (Berzins et al., 1999; Boursalian et al., 2004). Differential incorporation of TREC-rich RTEs into the general population in HIV-infected individuals might particularly occur in response to the preferential loss of CD4 T cells induced by the virus. We note that enhanced replacement of established, TREC-poor naive cells with TREC-rich RTEs in this case might increase TREC numbers even above their normal levels, as indicated in the study of Nobile et al. (2004).

Impeded thymocyte proliferation likely reduces thy-

mic output to an extent that depends on the nature of the actual compensatory mechanism, which is yet to be fully defined. An important implication of our analysis, in conjunction with earlier studies in mice (Berzins et al., 1999), is that peripheral sjTREC numbers are a direct (though not proportional) measure of the input of RTEs into the peripheral population of T cells, and these numbers progressively diminish during the chronic phase of HIV infection (Nobile et al., 2004). The decline in the input of new T cells might be detrimental to the patient, because it mimics the consequences of normal aging (Appay and Rowland-Jones, 2002) (Figure 3). Moreover, when coupled to other detrimental factors associated with HIV infection, including an accelerated loss of naive and memory cells through activation-induced differentiation (Grossman et al., 2002; Hazenberg et al., 2000a, 2000b), such a defect in thymic function might have greater, perhaps critical, consequences. Finally, proliferation of TCRβ⁺α⁻ precursor T cells facilitates increased TCR diversity by promoting the association of any given functional TCRβ chain with approximately 25 different TCRα chains (Arstila et al., 1999). Consequently, HIV infection, by forcing the generation of T cells with a limited intrathymic proliferation history, not only reduces RTE production but likely also their TCR diversity. In the long run, this may affect the naive T cell repertoire.

Under ART, both thymic function and RTE dynamics appear to have been normalized in certain, but not all, individuals. Surprisingly, older patients responded better to treatment than younger ones, because both the final sj/βTREC ratio (Spearman's, $r = 0.85$ and $p = 0.01$; Supplemental Figure S2A) and the fold variation of the sj/βTREC ratio (Spearman's, $r = 0.91$ and $p = 0.01$; Supplemental Figure S2B) increased with age. Perhaps the impact of primary infection on precursor T cell proliferation was stronger and less reversible in the younger, more active thymii. Alternatively, in younger patients, the rebound in thymopoiesis, if ever achieved, may necessitate restoration of thymic architecture and, therefore, a longer recovery time.

In summary, we have elaborated a method of measuring precursor T cell proliferation within the thymus using solely peripheral blood cells, enabling noninvasive assessment of thymic function independent of peripheral events. Specifically, we measure the mean number of cell divisions between DβJβ rearrangement and rear-

rangement at the α locus whereby the sjTREC is formed. We do this by quantifying the frequencies of D β J β TRECs and sjTRECs. The change in the sj/ β TREC ratio shows that primary HIV infection rapidly imposes, mainly in younger patients, a lasting thymic dysfunction characterized by impaired thymocyte proliferation. This thymic defect appears to be partly compensated in the periphery in the early phase of HIV infection through yet-unspecified mechanisms. The demonstrated ability to assess thymocyte proliferation by measuring the sj/ β TREC ratio may prove very useful in a wide variety of immunological settings especially when manipulation of thymopoiesis through immunotherapy becomes feasible.

Experimental Procedures

Patients and Control Individuals

Blood samples were obtained from 28 healthy volunteers and 27 HIV-1-infected patients enrolled during primary infection. A summary of the virological and immunological status of patients at baseline is shown in Supplemental Table S1. The patients were followed over a 2 year period. Ten patients started antiretroviral therapy either prior to enrolment ($n = 3$) or in the course of this study ($n = 7$). The remaining 17 individuals preferred not to receive ART but agreed to be followed. Clinical protocols conformed to ethical guidelines of the authors' institutions and human experimentation guidelines of the US Department of Health and Human Services. Samples were obtained with the informed consent of the subjects.

FACS Purification of Thymocyte Subsets

Thymic tissues (pediatric or adult) were sampled during surgery for major cardiopathy with the informed consent of the patients or of the child's parents and according to the guidelines of the bioethical committee of Centre Hospitalier de l'Université de Montréal (CHUM) or Hôpital de La-Pitié Salpêtrière, Paris.

Pediatric thymii: triple-negative (TN) thymocytes were purified through depletion of CD4- and CD8-expressing cells with magnetic beads (Miltenyi Biotec). TN thymocytes were then stained with directly conjugated anti-CD34-PE and anti-CD1a-FITC cell-surface antibodies. Thymocyte subsets were sorted on a FACS Vantage. ISP, DP, and SP thymocytes were FACS purified with directly conjugated anti-CD3-APC, anti-CD4-PE, and anti-CD8-FITC. Thymocyte subpopulation purity was always above 80%.

Adult thymii: after mechanical dissociation of thymic tissue with cell strainers (Falcon), isolated thymocytes were stained with (1) anti-CD34-FITC, anti-CD1a-PE, anti-CD4-CyChrome, anti-CD3-APC, and anti-CD8-APC or (2) anti-CD8-FITC, anti-CD3-PE, and anti-CD4-CyChrome (BD Pharmingen). The cells were then sorted with the MoFlo cell sorter (Cytomatrix).

In Vitro Stimulation

PBMCs distributed evenly in 24-well plates (Falcon) were cultured for 7 days in RPMI 10% FCS with 30 ng/ml anti-CD3 (OKT3) at 5×10^5 cells per well. IL-2 (30 pg/ml) was added 24 hr after stimulation. Cells were counted and harvested every 24 hr.

Ki67 Analysis

Thymii: after thymic tissue dissociation as described above, analysis was performed by surface staining cells for CD3/CD4/CD8 (BD Pharmingen) followed by fixation/permeabilization and intracellular staining for Ki67 (BD Pharmingen). Ki67 expression was measured in CD3⁺/lowCD4⁺CD8⁺, CD3⁺/lowCD4⁺CD8⁺, CD3⁺/lowCD4⁺CD8⁺, CD3⁺CD4⁺CD8⁺, CD3⁺CD4⁺CD8⁺, and CD3⁺CD4⁺CD8⁺ thymocytes. A minimum of 3×10^5 events in the live cell gate, as defined by forward and side scatter, was accumulated for each sample. Data were acquired on a FACSCalibur system and analyzed over a four log scale with CellQuest Pro software (Becton Dickinson Systems).

PBMCs: analysis was performed as described above with surface staining for CD3/CD45RA/CD27 (BD Pharmingen). Ki67 expression was measured in CD3⁺CD45RA⁺CD27⁺, CD3⁺CD45RA⁺CD27⁺, CD3⁺CD45RA⁺CD27⁺, and CD3⁺CD45RA⁺CD27⁺ cells.

TREC Quantification

Primers specific for the sjTREC (δ Rec- Ψ J α), each of the 10 D β J β TRECs (D β 1-J β 1.1 to D β 2-J β 2.4), and the human CD3 γ -chain gene were defined on the human germline sequence (Genebank accession numbers AE00061, U66061, and X06026; Supplemental Table S3). Parallel quantification of each deletion circle together with the CD3 γ amplicon was performed for each sample with the LightCycler technology (Roche Diagnostics). PBMCs were lysed in Tween-20 (0.05%), NP-40 (0.05%), and Proteinase K (100 μ g/mL) for 30 min at 56°C and then 15 min at 98°C. Multiplex PCR amplification was performed for sjTREC or each of the 10 D β J β TRECs together with the CD3 γ chain in 100 μ l (10 min initial denaturation at 95°C, 30 s at 95°C, 30 s at 60°C, and 2 min at 72°C for 22 cycles) with outer 3'/5' primer pairs. PCR conditions in the LightCycler experiments, performed on 1/100th of the initial PCR, were: 1 min initial denaturation at 95°C, 1 s at 95°C, 10 s at 60°C, and 15 s at 72°C for 40 cycles. Fluorescence measurements were performed at the end of the elongation steps. TRECs and CD3 γ LightCycler quantifications were performed in independent experiments but on the same first round, serially diluted standard curve. This highly sensitive nested quantitative PCR assay allows the detection of one copy out of 10⁵ cells for each DNA circle. The sjTRECs were quantified in triplicate, whereas duplicate experiments were performed for each individual D β J β TREC for all studied samples. The sum of D β J β TREC frequencies (D β J β TRECs) is calculated as 1.3-fold the sum of the ten measured D β J β TREC frequencies (in order to extrapolate to the 13 principal D β J β TREC). The sj/ β TREC ratio is the sjTREC frequency divided by the sum of D β J β TREC frequencies (sj/ β TREC = sjTRECs/D β J β TRECs).

Statistics

Statistical analysis (Mann-Whitney test, two-tailed Student's *t* test, Spearman's correlation test, *r*, and *p* values) was performed through the Vassar college website and StatView F-4.5. Student's *t* tests were performed on log-transformed values in order to analyze normally distributed populations of samples. Paired Student's *t* tests were used in Figures 1A, 1B, and 2C to demonstrate the reproducibility of the assays. All the other Student's *t* tests were unpaired. An *r* value of ≥ 0.3 or ≤ -0.3 and a *p* value of ≤ 0.05 were considered significant.

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